0026-895X/03/6303-722–731$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 63:722–731, 2003

Vol. 63, No. 3
2178/1047490
Printed in U.S.A.

Dehydroepiandrosterone Affects the Expression of Multiple Genes in Rat Liver Including 11β-Hydroxysteroid Dehydrogenase Type 1: A cDNA Array Analysis

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Received September 30, 2002; accepted November 27, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Dehydroepiandrosterone (DHEA) is a C-19 adrenal steroid precursor to the gonadal steroids. In humans, circulating levels of DHEA, as its sulfated conjugate, are high at puberty and throughout early adulthood but decline with age. Dietary supplementation to maintain high levels of DHEA purportedly has beneficial effects on cognitive memory, the immune system, and fat and carbohydrate metabolism. In rodents, DHEA is a peroxisome proliferator that induces genes for the classical peroxisomal and microsomal enzymes associated with this response. These effects are mediated through activation of peroxisome proliferator-activated receptor α (PPARα). However, DHEA can affect the expression of genes independently of PPARα, including the gene for the major inducible drug and xenobiotic metabolizing enzyme, cytochrome P450 3A23. To elucidate the biochemistry associated with DHEA treatment, we employed a cDNA gene expression array using liver RNA from rats treated with DHEA or the classic peroxisome proliferator nafenopin. Principal components analysis identified 30 to 35 genes whose expression was affected by DHEA and/or nafenopin. Some were genes previously identified as PPAR-responsive genes. Changes in expression of several affected genes were verified by quantitative reverse transcriptase-polymerase chain reaction. These included aquaporin 3, which was induced by DHEA and to a lesser extent nafenopin, nuclear tyrosine phosphatase, which was induced by both agents, and 11β-hydroxysteroid dehydrogenase 1, which was decreased by treatment with DHEA in a dose-dependent fashion. Regulation of 11β-hydroxysteroid dehydrogenase 1 expression is important since the enzyme is believed to amplify local glucocorticoid signaling, and its repression may cause some of the metabolic effects associated with DHEA.

Steroid hormones regulate gene expression by binding nuclear receptors associated with specific hormone-responsive elements on DNA and activating them to recruit coactivators and other components of the transcription machinery to target genes (Horwitz et al., 1996; McKenna et al., 1999; Klinge, 2000). Details of this process have been well described for hormonal steroids like estrogen, glucocorticoids, and androgens. However, much less information is available for the sterol found in highest circulating levels in humans, dehydroepiandrosterone (DHEA).

DHEA is a C-19 adrenal steroid precursor to estrogens and androgens. In humans it is found at micromolar levels in the circulation largely as the sulfate ester (Herbert, 1995). Plasma concentrations are highest between the ages of 20 and 30, decreasing thereafter with age (Orentreich et al., 1984). There are numerous reports that DHEA exerts protective effects in rodents against diseases such as cancer and diabetes and has anti-lipidemic effects, lowering levels of triglycerides and cholesterol (Schwartz, 1979; Coleman et al., 1982; Loria et al., 1988; Nestler et al., 1988, 1991; Ben-Nathan et al., 1992; Inano et al., 1995). In addition to these beneficial effects, DHEA is a potent peroxisome proliferator in rodents when administered at pharmacological doses; i.e., >5 mg/kg/day (Wu et al., 1989; Prough et al., 1994; Lubet et al., 1998). As such, it induces many of the same pathophysiological effects (liver damage and hepatocellular carcinoma) and biochemical effects (induction of peroxisomal and microsomal enzymes) as classic peroxisome proliferators, like clofibrate, the Wyeth compound (Wy-14,643), and nafenopin (Reddy and Krishnakantha, 1975; Gibson, 1993; Reddy and Mannaerts, 1994). Both DHEA and nafenopin induce peroxisome proliferation through activation of the nuclear receptor, PPARα (Peters et al., 1996, 1997; Gonzalez et al., 1998). However, not all the effects of DHEA are mediated strictly

This research was supported by the National Institutes of Health Grant DK54774. S.L.R. was supported by a National Research Service Award Fellowship F32 ES05927. S.G. was supported by a University Fellowship from the University of Louisville Graduate School.

ABBREVIATIONS: DHEA, dehydroepiandrosterone; PPAR, peroxisome proliferator-activated receptor; PRL1, phosphatase of regenerating liver 1; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; HPRT, hypoxanthine-guanine phosphoribosyl transferase; HSP, heat shock protein; PXR, pregnane X receptor; PCA, principal components analysis; RT-PCR, reverse transcriptase-polymer chain reaction.

through PPARα. For example, DHEA but not nafenopin induces the expression of *CYP3A23*, the major inducible xenobiotic metabolizing cytochrome P450 in rat liver (Singleton et al., 1999). This effect is most likely mediated by the pregnane X receptor (PXR), because activators for this receptor, including DHEA, have been shown to induce members of the CYP3A family (Kliewer et al., 1998; Lehmann et al., 1998; Savas et al., 2000; Ripp et al., 2002).

The current study was initiated to identify genes whose expression was affected by DHEA or nafenopin in rat liver. We used cDNA expression arrays to assess modulation of expression of nearly 1200 genes from control, DHEA- or nafenopin-treated rats, and principal components analysis (PCA) to examine the expression array data (Hilsenbeck et al., 1999). This robust statistical approach identifies outliers, those genes whose expression differs in response to a treatment, and does not rely on comparison with arbitrarily chosen housekeeping genes. It also allows comparison between multiple treatment regimens; in our case comparing untreated, DHEA-treated, and nafenopin-treated animals. Using this approach, we identified 30 to 35 candidate genes whose expression levels differed in rat liver in response to DHEA or nafenopin treatment. Some of these had previously been shown to respond to DHEA or nafenopin using single gene analysis. However, many of the 35 genes have not previously been identified as responding to one or the other treatments, and some represented genes that were not expected to be affected. Single gene analysis of several of these mRNAs by RT-PCR confirmed their altered expression levels.

Among the genes identified in this analysis was corticosteroid 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), a gene whose expression was decreased by DHEA and to a lesser extent nafenopin treatment. RT-PCR verified this decrease, and further studies indicated that its extent was dependent on the dose of DHEA given in the diet. 11β-HSD1 is an oxidoreductase capable of interconverting the active 11-hydroxylated forms of glucocorticoids and their inactive 11-keto metabolites. It is believed that one of its principal roles is reactivation of inactive corticosteroids locally in tissues, thus amplifying glucocorticoid action. Since many effects of glucocorticoids are opposite to those reported for DHEA treatment, particularly on gluconeogenesis and insulin sensitivity, there is a potentially important relationship between DHEA treatment and the decreased expression of 11β-HSD1 that we observed.

Materials and Methods

Animal Treatments. Male Sprague-Dawley rats (180–200 g; Hsd:SD) were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and acclimated to control diet (AIN-76; ICN Biomedicals, Cleveland, OH) for 3 days prior to treatment. Animals were then maintained on control diet alone or diet supplemented with 0.45% DHEA, or other amounts of DHEA as indicated for 5 days. A third group of animals was maintained on control diet and given daily i.p. injections of nafenopin (30 mg/kg in corn oil) for 4 days. Animals were sacrificed and livers were perfused with 0.9% saline, excised, and immediately frozen in liquid nitrogen and stored at −80°C.

RNA Extraction. Total RNA was extracted from individual rat liver using TRIzol reagent (Invitrogen, Carlsbad, CA). Approximately 100 mg of liver tissue was homogenized in 2 ml of TRIzol reagent, and total RNA was extracted according to the manufacturer's instructions. The RNA was ethanol precipitated and stored in −80°C for future analysis.

Hybridization to Atlas cDNA Expression Array. The Clontech Rat 1.2 cDNA array, containing 1200 named genes was used in these studies. ^{32}P-Labeled cDNA was synthesized from 2 μg of total RNA based on the user manual provided by BD Biosciences Clontech (Palo Alto, CA). The array was prehybridized for 30 min at 68°C, and then hybridized with the labeled cDNA at 68°C overnight. After several wash steps, the array was exposed to a PhosphorImager screen, and the signal was quantified with ImageQuant software (Amersham Biosciences Inc., Piscataway, NJ).

Data Collection and Statistics. A grid was applied to the phosphorimage for data collection. The grid was reapplied three times, and measurements were performed each time to minimize errors caused by misalignment. Peak values were averaged and log transformed for further analysis. The background in each grid was determined by the local histogram peak method (http://www.nhgri.nih.gov/DIR/LCG/15K/HTML/img_analysis.html). Because it differed for each grid in the array, local background subtraction was preferred. The maximal intensity in each grid subtracted by its local background was used as the signal in that grid.

Two-component analysis was performed by linear regression of the data points for untreated rat liver (average of two separate hybridizations) and either DHEA- or nafenopin-treated rat liver (Hilsenbeck et al., 1999). Multicomponent analysis was performed using the SPSS statistics package. Sets of data from untreated, DHEA-treated, and nafenopin-treated animals were entered, and using principle components analysis, four new axes were extracted. The first component, P1, was extracted to best explain the total variance in the original data sets. In other words, along the P1 direction the data has a maximal amount of variance. P2 was selected to be perpendicular to P1 and explain the maximal residual variance. P3 is perpendicular to both P1 and P2 and represented the maximal residual variance after removing the variance represented by P1 and P2. P4 was determined by finding a direction perpendicular to P1, P2, and P3. A transformation matrix was generated by the software package and the original data sets were transformed and standardized onto those new axes. Since P2, P3, and P4 all obey normal distribution, a statistically robust prediction of the 99% confidence range can be calculated based on the model of bi-normal distribution (when only P2 and P4 are considered) or a similar approach (when all three components are considered). Any points outside the 99% prediction range (in the case of P2 and P4, this is an oval) were considered as outliers, which show expression changes among different treatments.

Semiquantitive RT-PCR. RNA from three individual animals was pooled for RT-PCR analysis. The primers used are described in Table 1. Avian myeloblastosis virus reverse transcriptase was used to generate first strand cDNA using gene-specific antisense primers, and then PCR was performed using *Taq*DNA polymerase under

TABLE 1

Gene	Sequence
Aquaporin 3(s)	5′-GAGGCAGAGAATGTGAAGCTGG-3′
Aquaporin 3(a)	5′-TTCAGCCTGGAGAACAGTACAC-3′
Aquaporin 9(s)	5′-GAGCAAGCAGACCTTGGTGGAA-3′
Aquaporin 9(a)	5′-TGATCAGGAGGCCAATGTCAAC-3′
Myosin heavy chain(s)	5′-CTTACTGGCTTGGACTGAAGGTACG-3′
Myosin heavy chain(a)	5′-GGAATGGCTGACCAACACTAGAAGG-3′
PRL1(s)	5′-GCACTTCCTCTGTATCGCCTCA-3′
PRL1(a)	5′-CTGCGACACAATGGACAGCAAT-3′
11β-HSD1(s)	5′-GTGTCTCGCTGCCTTGAACTCG-3′
11β-HSD1(a)	5′-CAGGCAGGACTGTTCCAAGACC-3′
HPRT(s)	5′-CGAGATGTCATGAAGGAGATGG-3′
HPRT(a)	5′-GGAAGTGACAAATCTACCTGACG-3′
Chymotrypsinogen(s)	5′-GAGAACATCCAGGTCCTGAAGATCG-3′
Chymotrypsinogen(a)	5′-GACTCAGTTGGCTTCCAAGATCTGC-3′
Phospholipase A_2(s)	5′-CCAGTGGACGACTTAGACAGGTGCT-3′
Phospholipase A_2(a)	5′-CTTTATTAGAGGTGCGGTGCAGAA-3′

s, sense; a, antisense.

optimal amplification conditions determined for each primer set (Mg^{2+} concentration and annealing temperature). The amplification cycle was selected in the linear amplification range. This was determined for each target gene by amplifying 2 μg of RNA at different cycle numbers. The quantity of target RNA relative to that of a housekeeping gene was determined using an RNA concentration gradient to ensure a linear response of PCR product to RNA. Following PCR amplification, the products were separated on a 1% agarose gel, stained with ethidium bromide, and the pictures were digitized with a Hewlett-Packard office scanner and quantified using ImageQuant software. The initial slope was calculated and compared with the initial slope of a housekeeping gene used as the control for normalization and quantitation. The housekeeping genes were selected on the basis of their showing less than 2-fold changes with gene array analysis and their ability to be amplified in a linear range at the appropriate cycle number for the particular target gene.

Results

Comparison of Housekeeping Gene Hybridization on Rat Atlas 1.2 cDNA Arrays. We utilized Clontech rat atlas 1.2 membrane arrays to examine RNA from livers of untreated, DHEA-treated, and nafenopin-treated animals. The data were analyzed with a PhosphorImager, and visual inspection clearly shows genes whose level of expression changed. Two such genes, CYP4A3 and aquaporin 3, are highlighted in Fig. 1. Additional analysis and interpretation of these data presented an imposing problem. With classical single gene analysis, expression levels for genes of interest are standardized relative to a housekeeping gene, with the implicit assumption that the treatment does not affect expression of such genes. Clontech membranes contain a variety of housekeeping genes, which are located along the bottom row and separated from the other genes on the array (Fig. 1). Comparing one with another revealed that they were not always reliable controls. Quantitative comparison of the housekeeping gene expression indicated that the magnitude of changes in expression between untreated and treated samples differed among these genes (data not shown).

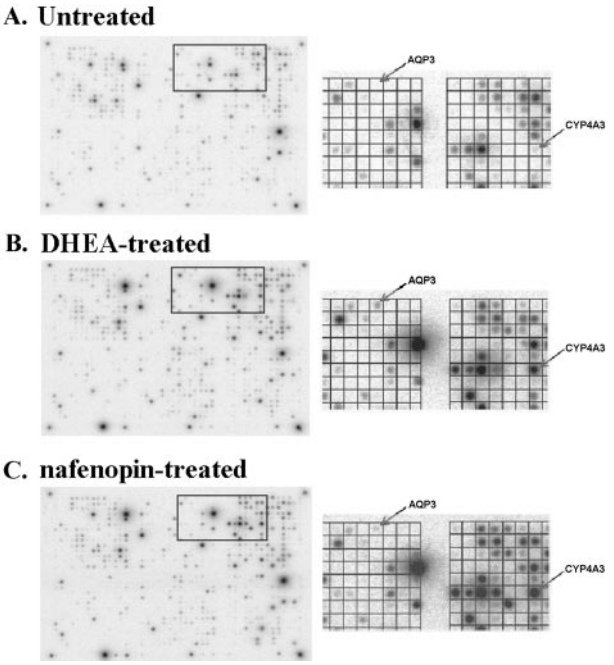

Fig. 1. DNA array hybridization with rat liver cDNA. Rat liver RNA from untreated, DHEA-, or nafenopin-treated animals was used to prepare ^{32}P-labeled cDNA, and hybridized with Clontech Rat 1.2 gene expression arrays as described under *Materials and Methods*. Membranes were exposed to a PhosphorImager screen for 7 days. One 14 × 14 grid was applied to each of the six blocks on the image with ImageQuant software. The single strip along the bottom of the filters are housekeeping genes, as designated by Clontech. Enlarged areas of the image are shown on the right, with spots for aquaporin 3 and CYP 4A3 indicated by arrows.

Principal Components Analysis of Array Data. Rather than comparing expression with a single housekeeping gene or an arbitrary group of housekeeping genes, we examined the array data by PCA as described under *Materials and Methods*. In PCA, each gene represents an independent observation providing almost 1200 separate observations and allowing a robust statistical analysis of the data. Data points were evaluated relative to the average expression of all genes with the implicit assumption that most of the nearly 1200 genes on the array did not change with the experimental treatment. This assumption is valid as long as the gene array is not restricted to genes whose expression changes. Two-component bivariate analyses (untreated and either DHEA or nafenopin treatment) are shown in Fig. 2, A and B. The data are plotted as untreated on the *x*-axis, DHEA, or nafenopin treatment on the *y*-axis. Linear regression was used to generate the solid diagonal line representing the average gene expression, with genes expressed at low levels represented by points closer to the origin, whereas

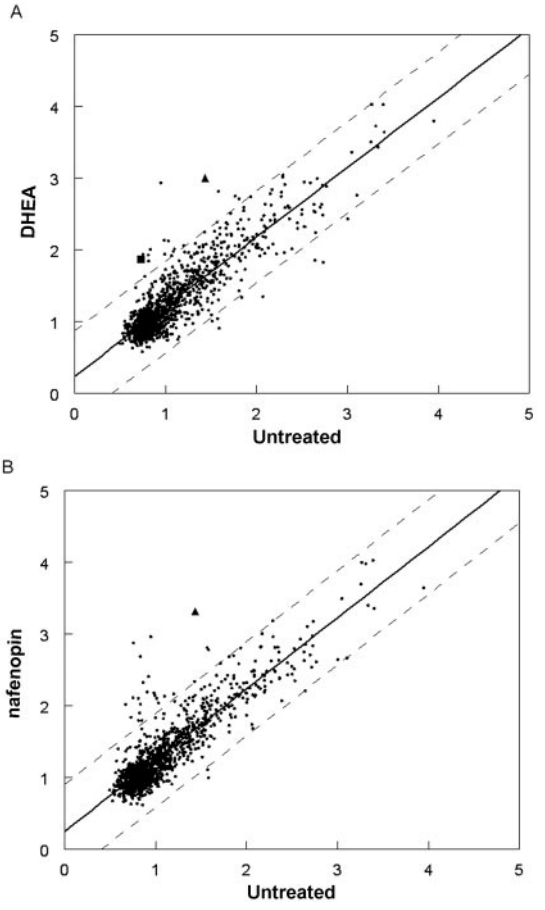

Fig. 2. Two-component analysis of array data. Quantitative values obtained from ImageQuant software were log-transformed and analyzed by linear regression; untreated versus DHEA-treated (panel A) and untreated versus nafenopin-treated (panel B). Solid lines are the average gene expression; dotted lines are the 99% confidence intervals from linear regression. ▲, CYP4A3; ■, aquaporin 3; ●, all other genes.

more highly expressed genes are further from the origin along the diagonal. The dotted line is the 99% confidence interval that genes did not vary from this average. Table 2 lists genes that are statistical outliers in the bivariate analysis of DHEA-treated versus untreated (Fig. 2A) or nafenopin-treated versus untreated (Fig. 2B). The list is organized as follows: 1) genes whose expression is known to be regulated by peroxisome proliferators; 2) genes whose expression is affected by both DHEA and nafenopin treatment but not previously identified as such; 3) genes whose expression is affected by DHEA more than nafenopin treatment; or 4) genes affected by nafenopin more than DHEA treatment. Within each group, genes are in rank order from the highest to the lowest fold change in expression. The fold changes between untreated and DHEA-treated or untreated and nafenopin-treated for this list of affected genes were 4.6 or greater. This number was not an arbitrarily chosen cut-off but established by the data and statistical analysis used to generate the 99% confidence interval. Several of the genes in Table 2 appeared as outliers only in bivariate comparisons between DHEA-treated and untreated or nafenopin-treated and untreated, but not both. Examination of the data indicated that expression of some of these genes appeared to vary with both treatments. However, the variation had only attained the stringent cut-off established by the 99% confidence interval for one of the treatments. These genes are indicated by an asterisk in Table 2. In these cases, the genes were still grouped as responding to both treatment with DHEA and nafenopin since the fold changes were similar. Genes in this group included: 3-keto-CoA thiolase, mitochondrial *O*-palmitoyltransferase I, HSP90, insulin-like growth factor-binding protein I, liver carboxylesterase 10, corticosteroid 11-β-hydroxysteroid dehydrogenase I, and cytochrome P450 2C11.

Multicomponent Transformation of the Array Data. Transformation of these data to a multicomponent system allows the expression of genes from two separate controls, DHEA and nafenopin treatment, to be directly compared. This analysis centers data sets around an overall mean and uses a variance-covariance matrix to extract new components or axes, P1, P2, P3, and P4. Multiplying the PhosphorImager density data by a coefficient chosen to minimize the variance generates these components. The goal of this approach is to identify statistical outliers representing genes whose expression varies with the treatments. The principal components themselves may not have a strict physical meaning, since they were constructed simply to minimize variations in the data. However, by understanding the source of variation for each component, a general understanding of their meanings can be inferred. P1 is the maximum amount of variance in all genes, regardless of treatment condition and represented 90.1% of the total variance in expression of all genes. Removing the variance due to P1 eliminates differences in expression levels of different genes. By eliminating this variance, all genes whose expression is not influenced by the treatments fall at the center of the graph in Fig. 3, regardless of their overall levels of expression. Component P2 is largely the variance due to the effects of DHEA or nafenopin treatment and explains 4.7% of the total variance in the data set. P2 is the *x*-axis in Fig. 3. The remaining variance is due to P3 (3.1%) and P4 (2.1%), with P3 largely representing differences between the two controls and P4 the differences be-

TABLE 2
List of affected genes from two-component analysis

DHEA vs. Untreated	Fold Change	Nafenopin vs. Untreated	Fold Change
Gene expression known to be affected by treatment with peroxisome proliferators		Gene expression known to be affected by treatment with peroxisome proliferators	
Cytochrome P540 4A1	60.6	Cytochrome P450 4A1	59.2
Cytochrome P450 4A3	23.7	Cytochrome P450 4A3	44.3
Acyl-CoA oxidase	11.1	Acyl-CoA oxidase	9.1
Fatty acid transport protein	8.1	Fatty acid transport protein	11.3
NADPH-cytochrome P450 reductase	7.8	NADPH-cytochrome P450-oxidoreductase	5.9
3-Keto-CoA thiolase*	4.3	3-Keto-CoA thiolase	10.1
Cytochrome P450 2C11	−5.2	Cytochrome P450 2C11*	−3.8
Corticosteroid 11-β-dehydrogenase 1	−7.9	Corticosteroid 11-β-dehydrogenase 1*	−4.2
Apolipoprotein A–IV	−8.9	Apolipoprotein A–IV	−4.6
Additional genes - expression affected by DHEA and nafenopin treatment		Additional genes - expression affected by DHEA and nafenopin treatment	
Acetylcholine receptor, nicotinic, α4	9.4	Acetylcholine receptor, nicotinic, α4	13.1
Androgen binding protein	9.1	Androgen binding protein	8.8
Arrestin D + guanine aminohydrolase	8.8	Arrestin D + guanine aminohydrolase	9.0
Antigen peptide transporter 2	6.6	Antigen peptide transporter 2	5.5
Liver carboxylesterase 10	5.2	Liver carboxylesterase 10*	4.3
Insulin-like growth factor binding protein 1	4.9	Insulin-like growth factor binding protein 1*	3.1
Cytochrome P450 17	4.6	Cytochrome P450 17	5.3
Mitochondrial carnitine *O*-palmitoyltransferase I*	4.2	Mitochondrial carnitine O-palmitoyltransferase I	4.7
HSP84; HSP90-β*	3.6	HSP84; HSP90-β	4.6
Plasma membrane Ca + 2 ATPase	−5.2	Plasma membrane Ca + 2 ATPase	−5.0
Gene expression affected by DHEA- more than nafenopin-treatment		Gene expression affected by nafenopin more than DHEA treatment	
Aquaporin 3	8.5	Bile-salt-activated lipase	74.7
Nuclear tyrosine phosphatase	6.3	Trypsinogen II	40.9
A-raf	−4.8	Triacylglycerol lipase	17.4
β2-microglobulin	−11.5	Chymotrypsinogen B	16.6
		Elastase 2	12.7
		Phospholipase A2	11.0
		Colipase	7.1
		P55cdc: cdc20	6.8
		Serum/glucocorticoid-regulated Ser/Thr protein kinase	−6.6

*Variation at the 99% confidence interval with only DHEA treatment or nafenopin treatment, but not both.

tween DHEA- and nafenopin-treated samples. The graph shown in Fig. 3 plots P4 on the *y*-axis and P2 on the *x*-axis. The 99% confidence interval for invariant genes is represented by the outer oval and the 95% confidence interval by the inner oval. Points inside the oval are genes whose expression does not statistically vary among the three conditions, whereas those outside the oval are statistical outliers, representing genes whose expression varies among samples from untreated, DHEA-treated, and nafenopin-treated rats. The points to the left of the central vertical line on the *x*-axis are genes whose expression is repressed by DHEA or nafenopin treatment, whereas those to the right are genes whose expression is induced. Similarly, points above the central horizontal line on the *y*-axis are transcripts expressed to a greater extent in DHEA than nafenopin treatment, whereas those below the axis are expressed to a greater extent in nafenopin than DHEA treatment.

To generate a list of genes potentially regulated by either DHEA or nafenopin by this analysis, we eliminated those whose change in expression was due to component P3, since they would largely have been due to differences between the controls. Hughes et al. (2000) identified genes that differed in expression among controls in array data from yeast and suggested that their expression might be highly variable independent of any treatment. They also cautioned against including such genes in any list of those that vary with a particular condition or treatment regimen. For the sake of comparison, we did generate a list of outliers including those contributed by P3. Five out of six of the additional genes on this list were also identified as outliers in bivariate analysis between the two controls, confirming that P3 represented contributions from variations in the controls. Among these were H-ras, HSP90, ceruloplasmin, insulin-like growth factor I, macrophage migratory inhibitory factor, and mitogen-activated protein kinase p38.

Genes Identified As Outliers between Control, DHEA, and Nafenopin Treatment. Figure 4 is a graphical representation of the outlier genes. The data are represented as a bar graph with values expressed on a log to the base 2 scale, with actual fold changes indicated on the axis. The genes are arranged in the same groupings used in Table 2. The group of known PPARα-responsive genes include several of the classical peroxisome proliferator-induced genes such as CYP4A1, CYP4A3, fatty acid transport protein, and acyl-CoA oxidase. There were several other genes whose expression levels varied with both DHEA and nafenopin treatment, which have not previously been identified as peroxisome proliferator or PPARα-responsive. For example, androgen-binding protein, also known as sex hormone binding globulin, was induced over 8-fold by both DHEA and nafenopin treatment, and expression of plasma membrane Ca^{2+}-ATPase was substantially decreased. There were a few genes whose expression was affected more by DHEA than nafenopin treatment. This list includes aquaporin 3, which was induced 9.2-fold by DHEA but not nafenopin treatment, nuclear tyrosine phosphatase (PRL1), an early response gene to liver regeneration (Diamond et al., 1994) that was induced 4.4-fold by DHEA treatment, and β-2 microglobulin, which was decreased 20-fold by DHEA treatment. Finally, there were a number of genes whose expression was affected by nafenopin treatment to a greater extent than DHEA treatment. Most of these were pancreatic genes including bile salt-activated lipase, trypsinogen II, chymotrypsinogen, triacylglycerol lipase, elastase, phospholipase A2, and colipase. Semiquantitative RT-PCR analysis of two of these, chymotrypsinogen and phospholipase A2, confirmed that they were expressed in liver from nafenopin-treated but not control or DHEA fed animals (Fig. 5A).

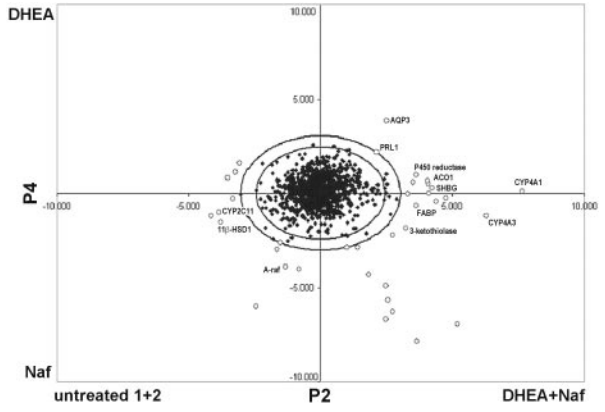

Fig. 3. Multicomponent analysis. Four sets of data from untreated (2), DHEA-, or nafenopin-treated animals were analyzed by principal components analysis with SPSS software (see *Materials and Methods*). Two of the resulting principle components are plotted, P2 on the *x*-axis and P4 on the *y*-axis. P2 is related to variance in the data resulting from treatment with either DHEA or nafenopin; P4 is related to variance in the data between DHEA and nafenopin treatment. The 99% confidence interval that a data point does not differ from the global average is represented by the outer oval, and a 95% confidence interval is represented by the inner oval. ●, data points for genes within the 99% confidence interval; ○, data points for outliers. Names for some of the outlier genes are indicated.

The 99% confidence interval ($p < 0.01$) for the multicomponent analysis is a statistically robust criterion for genes whose expression has changed. No genes with fold changes less than 5.4 qualified as outliers in this analysis. There were, however, several genes whose change in expression placed them right on or close to that 99% line. Therefore, we determined which genes were outliers within the less robust 95 to 99% confidence interval (inner circle in Fig. 3). A list of these eleven additional genes appears in Table 3. Four of the eleven (marked with an asterisk) also appeared on the single bivariate comparisons in Table 2.

There were several candidate genes that appeared as statistical outliers in multicomponent analysis but did not seem to be expressed at all upon visual inspection of the arrays. Most of these were adjacent on the array to a highly expressed gene or a gene whose expression varied dramatically with one treatment or the other. For example, the grid position immediately to the left of CYP4A3 in Fig. 1 is ceramide UDP-galactosyltransferase. In these cases, the overflow of signal from the adjacent expressed gene appeared to create a false positive. Because the number of total outliers was relatively small, these could be easily identified by visual inspection. Table 4 is a list of those reported as probable false positives.

Confirmation That Gene Expression Was Affected by DHEA or Nafenopin Using RT-PCR: Aquaporin 3. We selected several potentially important target genes in the physiological and biochemical response to DHEA for further examination by independent single gene analysis. Aquaporin 3 was induced significantly by DHEA but not nafenopin treatment (Fig. 4). The literature describes aquaporin 9 as the primary isoform expressed in the liver whereas aqua-

porin 3 is reportedly not expressed (Frigeri et al., 1995; Tsukaguchi et al., 1999). We devised PCR primers specific for aquaporins 3 or 9 using regions of their respective mRNAs that were not conserved. The cycle number to obtain a linear response between PCR product and input RNA was determined, and subsequent amplification was performed at that cycle number with different initial RNA concentrations to assure a linear response to RNA. The PCR products for a single concentration of RNA from untreated, DHEA-, and nafenopin-treated animals are shown in Fig. 5. For quantitation, the data from several RNA concentrations were plotted and the initial slopes used to estimate the amount of PCR product. These values were normalized to the amount of myosin heavy chain transcript and plotted as a bar graph. The results demonstrate that aquaporin 9 mRNA is present in livers from untreated, DHEA-, and nafenopin-treated animals. However, its pattern of expression is not consistent with the array results. In contrast, aquaporin 3 mRNA is clearly present in livers from DHEA-treated animals, expressed to a lesser extent in nafenopin-treated animals, and absent in RNA from untreated animals. These data are entirely consistent with the array analysis both qualitatively and quantitatively (Figs. 1 and 4) and demonstrate that aquaporin 3 is indeed induced in the liver by treatment with DHEA and to a lesser extent with nafenopin.

PRL1 Is Induced by Treatment with DHEA. PRL1 is a nuclear phosphotyrosine phosphatase shown to be an early response gene in liver regeneration (Diamond et al., 1994). The array analysis indicated that PRL1 expression was increased in livers from DHEA-treated animals (4.4-fold) but only modestly increased in nafenopin-treated animals (1.6-fold). Results of RT-PCR analysis using HPRT as a control are shown in Fig. 5, and show a 4.1-fold increase in PRL1 transcript with DHEA treatment and a 3.16-fold increase

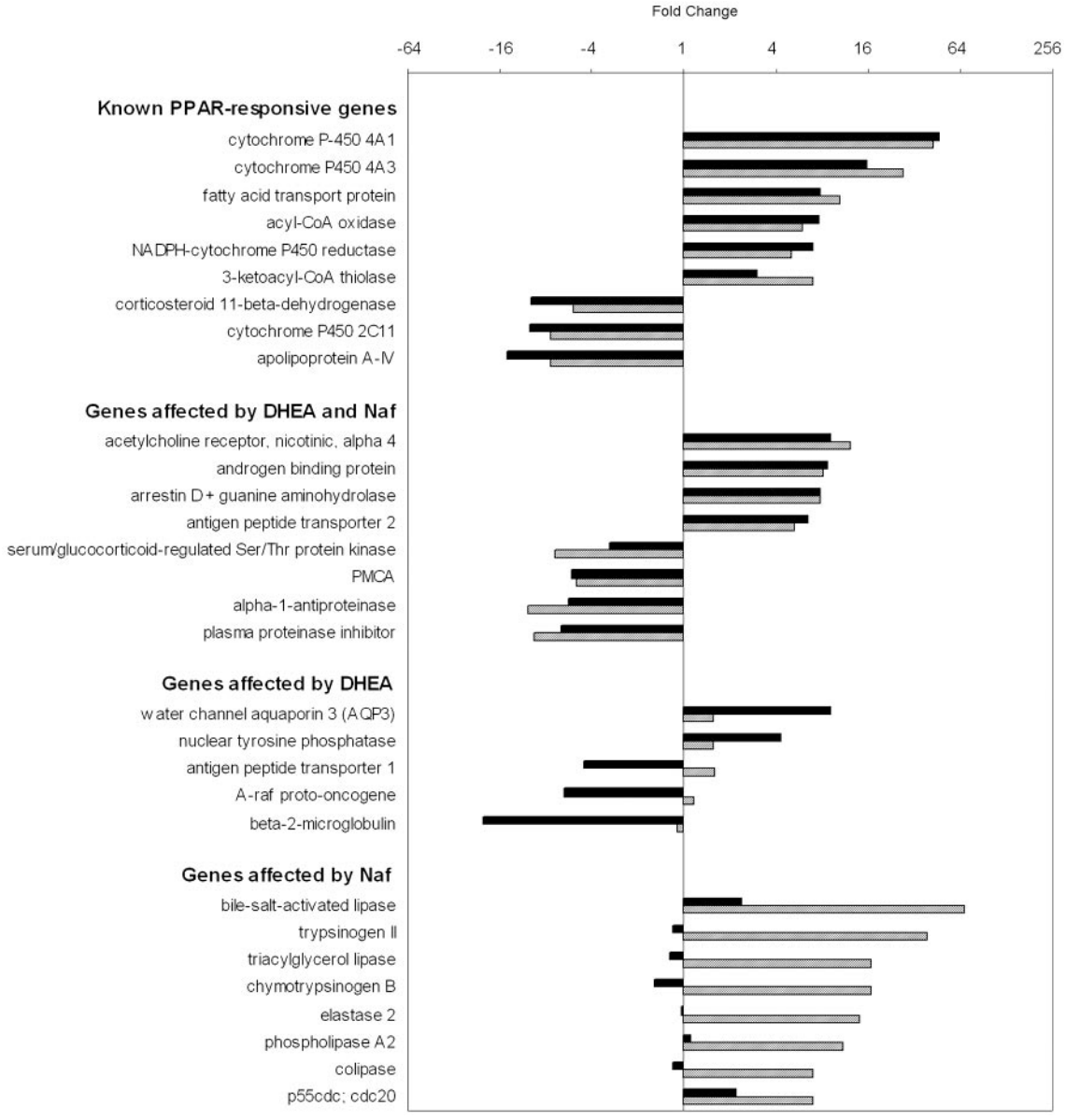

Fig. 4. Quantitative estimates of outlier genes. Genes determined by principal components analysis to be outliers were arranged in the indicated groupings. The axis is labeled as actual fold change, with values represented on a $\log_2$ scale. Solid bars, values from DHEA-treated animals; hatched bars, values from nafenopin-treated animals. Within each grouping, genes are arranged in relative order of the highest to the lowest fold change.

with nafenopin. These results agree qualitatively with the array analysis, although the fold change in PRL1 in nafenopin-treated animals is larger than predicted.

11β-HSD1 Is Decreased by DHEA and Nafenopin Treatment. An important enzyme in the prereceptor amplification of glucocorticoid signals is 11β-HSD1. It functions in tissues as an oxidoreductase to regenerate active glucocorticoids from their inactive 11-keto metabolites. Recent studies have shown that this transcript is down-regulated by the peroxisome proliferator Wy-14,643 and agonists of liver X receptor (Hermanowski-Vosatka et al., 2000, Stulnig et al., 2002). Array analysis had indicated 10- and 5.3-fold reductions in 11β-HSD1 transcript with DHEA and nafenopin treatments, respectively. Quantitative RT-PCR using HPRT as housekeeping gene are shown in Fig. 5 and indicate a 4-fold reduction in this transcript with both treatments. These data are consistent with results from the array analysis and the literature.

To determine whether the effect of DHEA on 11β-HSD1 was dose-dependent, animals were fed diets containing different amounts of DHEA. After 5 days, liver RNA was isolated and levels of 11β-HSD1 mRNA were determined by RT-PCR compared with that of control animals fed a normal diet. The data in Fig. 6 show that the decrease in 11β-HSD1 mRNA was dependent on the dose of DHEA, with significantly less RNA at the two highest doses used.

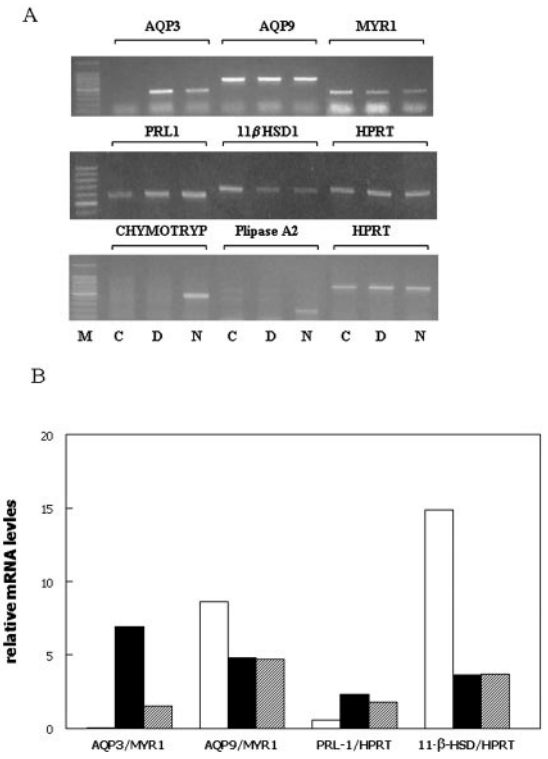

Fig. 5. RT-PCR analysis of selected genes. Quantitative RT-PCR was performed with pools of RNA (2 μg) from three separate treated animals, as described under *Materials and Methods*. Panel A, gel analysis of products from untreated (C), DHEA- (D), or nafenopin-treated (N) animals are shown along with a molecular weight marker lane (M). Panel B, RNA concentrations from 0.5 to 4 μg were subjected to RT-PCR, and the resulting gels scanned and quantified with ImageQuant software. Values were obtained from the initial slope of a graph of signal versus RNA concentration and are represented as mRNA levels relative to an appropriate housekeeping gene control, either myosin heavy chain (MYR1) or HPRT. Open bars, values from untreated animals; solid bars, values from DHEA-treated animals; hatched bars, from nafenopin-treated animals.

Discussion

We have used cDNA arrays to examine expression of rat liver genes in response to DHEA and nafenopin treatments. Nafenopin is a classic peroxisome proliferator and induces expression of a number of PPARα-responsive genes (Wu et al., 1989; Prough et al., 1994; Singleton et al., 1999). DHEA is also a peroxisome proliferator but may regulate genes unrelated to peroxisome proliferation (Singleton et al., 1999). Our major interest was to identify candidate genes regulated by these two agents. Simple analysis of array data by comparing expression levels to housekeeping genes can be misleading since not all housekeeping gene expression is invariant. In our analysis, some of the commonly used housekeeping genes differed between the untreated and treated samples and more importantly differed between DHEA and nafenopin treatment. In fact, phospholipase A2, designated as a housekeeping gene on the Clontech membrane, was clearly induced by nafenopin but not by DHEA treatment. PCA avoids the use of arbitrarily selected housekeeping genes, by comparing the expression of any particular gene with the average expression of all genes between treatments (Hilsenbeck et al., 1999). It is a robust statistical approach to the analysis of array data, identifying genes whose expression levels statistically outlies the norm, thus suggesting that their expression is affected by the treatment regimen. The implicit assumption is that most genes on the array do not vary in expression with a particular treatment, a reasonable assumption since the Clontech Rat 1.2 array is a general array, not selected to contain only targeted respon-

TABLE 3
Outlier genes from multicomponent analysis that fall between the 95 and 99% confidence interval

Gene	DHEA/Untreated	Nafenopin/Untreated	*p* Value
		-fold change	
Cytochrome P450 2C7	−5.3	−5.4	0.0103
Cytochrome P450 17*	3.9	4.5	0.014
Insulin-like growth factor binding protein 1*	4.5	2.8	0.026
Cathepsin c; cathepsin J	−2.3	−4.9	0.027
Carnitine *O*-palmitoyltransferase 1*	3.3	3.6	0.033
Liver carboxylesterase 10*	3.5	2.8	0.037
Fibroblast ADP/ATP carrier protein	1.4	−2.5	0.038
Serine/threonine kinase (PCTK2)	2.6	−1.3	0.039
Glycine receptor α3	4.2	3.1	0.040
Monocarboxylate transporter 1	3.15	3.5	0.043
HSP60	2.9	2.9	0.048

*Also appear as outliers on bivariate comparison list (Table 2).

sive genes. PCA can also compare multiple treatments. We used two-component analysis (untreated/DHEA or untreated/nafenopin), and multicomponent analysis (untreated/DHEA/nafenopin) to examine differences between genes expressed in DHEA- or nafenopin-treated rat liver. The lists of potentially regulated genes derived from these analyses were similar but not identical (Table 2 and Fig. 4). Most of the differences were in genes whose expression levels were nominally changed, such that they lay just outside the 99% confidence interval in one analysis, but just within that interval in the other. Genes on the bivariate comparison list that were not on the multicomponent list included liver carboxylesterase 10, insulin-like growth factor-binding protein 1, cytochrome P45017α, mitochondrial carnitine *O*-palmitoyltransferase 1, and HSP90. The first four were on the multicomponent list with less robust statistical criteria (Table 3). HSP90 was eliminated from the multicomponent list as a contribution from P3, representing genes that show large variation between controls. Three additional genes, antigen peptide transporter 1, α-1-antiproteinase, and plasma proteinase inhibitor, appeared on the multicomponent but not the bivariate comparison list. There were several probable false positives on the original list of outliers (Table 4) that appeared on the list because of their close proximity on the array to highly expressed and/or moderately expressed but highly regulated genes. The ImageQuant software detected this signal spillover and assigned a value above background for these genes, which tended to be highly variable accounting for their appearance on lists of outliers. For the most part, they could be easily eliminated by visual inspection.

The analysis identified several genes previously shown to be regulated by DHEA and nafenopin (Table 2 and Fig. 4A). These were primarily genes regulated by PPARα and involved in the peroxisome proliferation response. They included members of the CYP4A family of fatty acid ω-hydroxylases, fatty acyl-CoA oxidase, fatty acid transport protein, and other enzymes involved in peroxisomal fatty acid oxidation. The degree of induction determined by array analysis was similar to that reported previously using Northern blotting (Wu et al., 1989; Prough et al., 1994; Singleton et al., 1999).

In addition to genes known to be regulated by peroxisome proliferators, we identified several genes whose expression was affected to the same degree by DHEA and nafenopin treatment but have not previously been identified as peroxisome proliferator-responsive (Fig. 4B). Among them were four genes whose expression increased: acetylcholine receptor, androgen-binding protein (also known as sex hormone binding globulin), arrestin D/guanine aminohydrolase, and antigen peptide transporter 2. There were also four genes whose expression decreased, plasma proteinase inhibitor, α-1-antiproteinase, plasma membrane Ca^{2+} ATPase, and serum/glucocorticoid-regulated Ser/Thr protein kinase. The latter gene responds to glucocorticoids, and its down-regulation may reflect lower levels of corticosterone in the liver resulting from decreased expression of 11β-HSD1 (see below).

Multicomponent analysis can identify genes whose expression differs between several treatments, with a single robust statistical analysis. With this approach, we identified eight genes that showed moderate to dramatic increases in expression after treatment with nafenopin but not DHEA (Fig. 4C). Most of these were pancreatic genes. That such genes were expressed at all in the liver is perplexing. A review of the literature finds few references to pancreatic genes aberrantly expressed in the liver, although there is a report of immunoreactive material for pancreatic digestive enzymes in liver biopsies from patients with various liver cancers (Terada and Nakanuma, 1996). It is conceivable that this aberrant expression is diagnostic of liver damage, a hallmark of peroxisome proliferation. That these were seen with nafenopin but not DHEA treatment further suggests that DHEA, while inducing peroxisome proliferation, does not cause as much liver damage as nafenopin.

We also identified five genes regulated by DHEA treatment to a greater extent than nafenopin treatment (Fig. 4D). Expression of two of these, nuclear tyrosine phosphatase (PRL1) and aquaporin 3, was increased whereas expression of three others, antigen peptide transporter 1, A-raf, and β-2 microglobulin, was decreased. Aquaporin 3 is a member of a family of water channel proteins expressed in a tissue-specific manner (Frigeri et al., 1995; Tsukaguchi et al., 1999). According to the literature, aquaporin 9 not 3 is the liver isoform. Thus, we initially suspected that the array had misidentified the correct isoform. Using RT-PCR, we demonstrated that aquaporin 9 was expressed in liver from untreated, DHEA-, and nafenopin-treated animals; however, its pattern of expression was not in agreement with the array data. In contrast, aquaporin 3, although not observed by RT-PCR in livers of untreated animals, was clearly present in livers from DHEA-treated and to a lesser extent nafenopin-treated animals (Fig. 5). This pattern agreed with the array data and demonstrates that the array analysis had identified the correct aquaporin transcript. Aquaporin 3 belongs to the aquaglyceroporin subclass of water channels that allow movement of glycerol and other small nonpolar organic molecules into and out of the cell (Engel et al., 2000). As such, its induction may have important physiological consequences in helping maintain the osmotic balance in cells undergoing increased peroxisomal fatty acid oxidation and peroxisome proliferation. These target genes will be of considerable interest in trying to understand the physiological effects of DHEA and nafenopin in the liver. However, it remains to be seen whether they are part of the primary response to these agents or a secondary effect resulting from pathophysiology associated with peroxisomal proliferation.

One of the most intriguing genes identified by the array analysis was glucocorticoid 11β-HSD1. This enzyme is an oxidoreductase thought to reactivate glucocorticoids from their inactive keto metabolites. It is considered an amplifier of glucocorticoid action and has recently been shown to be the primary determinant of visceral obesity (Masuzaki et al., 2001). 11β-HSD1 expression was significantly reduced in response to the highest dose of DHEA (40% of the control value) but only marginally reduced in response to lower doses (Fig. 6). 11β-HSD1 has been shown to negatively respond to the peroxisome proliferator Wy-14,643 in liver and PPARγ ligands (rosiglitizone and thiazolidinedione) in adipose tissue (Hermanowski-Vosatka et al., 2000; Berger et al., 2001). It also responds negatively to liver X receptor agonists in both liver and adipose (Stulnig et al., 2002). Interestingly, the 11β-HSD1 knockout mouse shows similar phenotypic characteristics to those reported for DHEA supplementation, namely, increased insulin sensitivity, lowered serum cholesterol and triglycerides, decreased stress-induced hyperglycemia, and increased cognitive memory (Sandeep and Walker, 2001). It is possible that some of the metabolic effects associated with DHEA

TABLE 4
Probable false positives

Probable False Positives	Array Position	Interfering Gene (Position)
Cyclin E	A07d	β2-microglobulin (A08d)
Pro-thymosin α	A08e	β2-microglobulin (A08d)
HSP 27	A13j	HSP 84; HSP 90β (A13k)
Proton-gated cation channel DRASIC	B04k	Na^{2+} channel SCNβ2 (B05k)
Glucose transporter, sodium-dependent	B09b	ADP/ATP carrier protein (B08b)
Ceramide UDP-galactosyl transferase	C06f	CYP4A3 (C07f)
3-Oxo5-α-steroid 4-dehydrogenase	C06m	CYP4A1 (C07m)
Mast cell protease	F07n	Elastase 2 (F06n)
Leukocyte common antigen-related Tyr phosphatase	F12a	α-1 antitrypsin (F11b)
Phosphotyrosine phosphatase 6	F12b	α-1 antitrypsin (F11b)
O-6-Methylguanine-DNA methyltransferase	F14g	Orphan nuclear receptor TR4 (F13f)

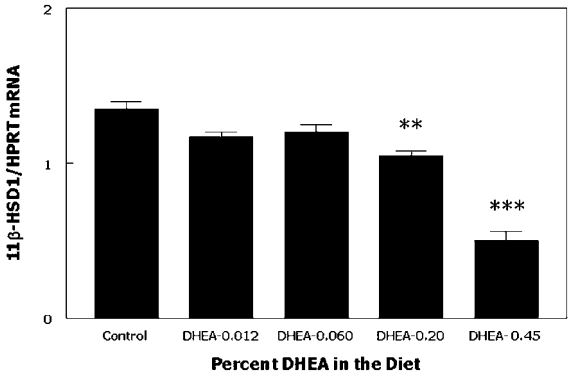

Fig. 6. Dose response of 11β-HSD1 mRNA to DHEA. Animals were fed diets containing 0.012, 0.06, 0.2, or 0.45% DHEA for 5 days. Liver RNA was prepared from three animals in each group, then pooled and subjected to quantitative RT-PCR. The results are expressed as 11β-HSD1 mRNA levels relative to those for HPRT. Results are the average of three independent measurements, and error bars represent standard errors of the mean. Statistical analysis was performed by Student's *t* test, and the significant difference from control are indicated as follows: $p < 0.01$ (**) or $p < 0.001$ (***).

supplementation are related to reduced expression of 11β-HSD1 and a subsequent decrease in local levels of active glucocorticoids. One of the other genes showing decreased expression in response to DHEA was glucocorticoid-regulated, Ser/Thr protein kinase (Fig. 4). This gene is glucocorticoid-responsive and may be decreased in response to lower corticosterone levels in the liver, resulting from decreased 11β-HSD1 gene expression.

The cDNA expression array analysis identified genes whose expression was affected by DHEA treatment, nafenopin treatment, or both. Some of these may be related to peroxisome proliferation since both agents are peroxisome proliferators. However, DHEA is known to have effects independent of peroxisome proliferation. Our previous studies showed that *CYP3A23* was induced in rat liver by DHEA but not nafenopin treatment (Singleton et al., 1999). *CYP3A23* contains a PXR-responsive element, and Ripp et al. (2002) have recently shown that DHEA and some of its metabolites can activate the murine PXR in cell-based reporter assays. It will be interesting to see whether other nuclear receptors can regulate the expression of the genes we have found to be affected by DHEA treatment.

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